

'NEW' GENES FROM 'NOVEL' PLANTS FOR ALTERING LIGNIFICATION

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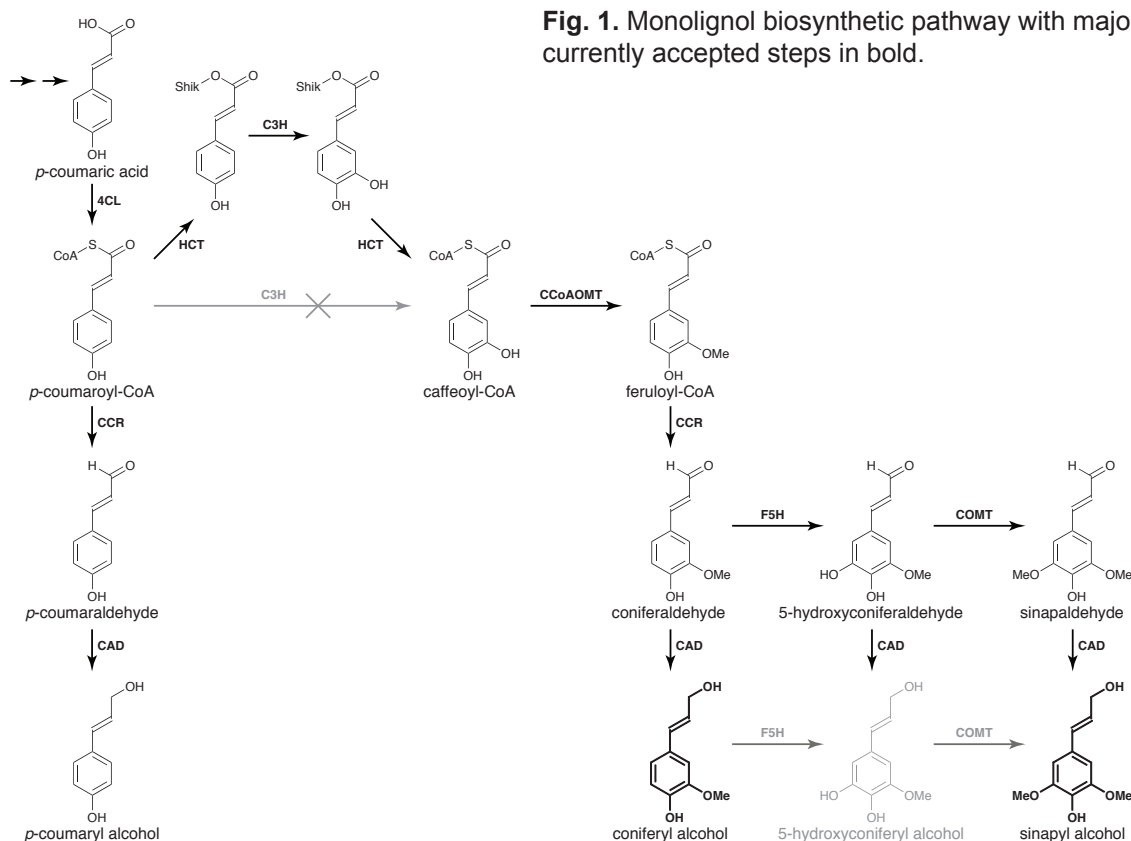
ABSTRACT

A promising approach toward addressing the biomass conversion recalcitrance problem, inherent in dealing with lignified plant cell walls, is by altering lignin composition and structure via plant engineering. One intriguing method is to introduce readily cleavable linkages into the backbone of the lignin polymer so that it can be more facilely depolymerized. Nature is providing clues to how this might best be accomplished. A series of transferase genes are already implicated in lignification in some plants, and plants are already capable of biosynthesizing conjugates, monolignol ferulates, that might be targeted to lignification. Here we describe these 'new' transferases, and the search for their genes, and begin to explore perhaps the most significant lignin alteration to date, that of creating 'zip-ligninTM' polymers *in planta*. Such modifications have the potential to revolutionize biomass conversion to pulp and paper or liquid biofuels and chemicals.

Keywords: Lignin; monolignols; lignin monomers; transgenics; transferases

INTRODUCTION

It has become increasingly clear over the past decade that lignins are NOT just polymerized from the three traditional monomers, the monolignols *p*-coumaryl, coniferyl, and sinapyl alcohols [1-7]. In addition to the massive lignin compositional changes that result from misregulation of monolignol pathway genes, more severe structural variations can be found. These include a variety of other phenolic compounds that should be recognized as authentic monomers in a range



of 'normal' plants. Add to this the ever increasing array of phenolic compounds that get recruited for, or perhaps simply end up inadvertently in, lignification in other mutants and transgenics. Although this would appear to, and does, add considerable complexity to the composition and structure of the resultant lignin, the generation of the polymer is delightfully consistent and predictable (to the extent that coupling and cross-coupling propensities of the various monomers are known). Each new monomer discovery adds more options to our arsenal for altering plant lignification to improve biomass conversion in industrial processes such as chemical pulping and chemical/biological biomass conversion to liquid fuels and chemicals, as well as for certain natural processes like ruminant digestibility. Some of the possibilities can be gleaned from nature herself and by observing how plants respond to up- and down-regulation of pathway genes. These strategies offer the potential to significantly improve our ability to efficiently and sustainably utilize our valuable plant resources.

Compositional Changes

Misregulation of monolignol pathway hydroxylase genes in particular, Figure 1, can produce striking compositional changes in the otherwise 'normal' lignin. Thus, the S/G composition is profoundly alterable, beyond the bounds found to date in 'natural' plants, by misregulation of *F5H* (that has more recently been shown to operate preferentially on coniferaldehyde and is therefore sometimes referred to as *CAld5H*) [8-11]. *F5H*-deficient mutants of *Arabidopsis* have G-only lignins whereas, quite amazingly, *F5H*-upregulation can be strikingly effective at shunting nearly all of the pathway toward sinapyl alcohol and therefore toward S lignin – *F5H*-upregulated poplar is some 97% S, as determined by NMR, implying an almost 40:1 S:G (although the ratio obviously becomes unreliable at such low G levels); natural plants top out at about 7:1 S:G. Analogously, the 3-hydroxylase, C3H, dramatically affects the H lignin content. Thus, the C3H-deficient *Arabidopsis ref8* mutant has no discernible G or S units and its lignin is solely composed of H-units [12]. The plant is particularly stunted, but it is not clear that the stunting is caused by the high-H lignin *per se*. Downregulation of the *C3H* gene, or the accompanying *HCT*, also

causes an increase in H levels in medicago, poplar, and pine [13-19]. A hydroxylase identified in the ancient plant selaginella, which predates both softwoods and hardwoods, is a dual-function hydroxylase capable of both 3- and 5-hydroxylation [20, 21]; when the selaginella gene is introduced into a C3H-deficient *ref8* mutant of Arabidopsis, the plant makes an unusual H-S lignin with only a very low G component.

Non-traditional Monolignols in Lignins

An increasing array of monomers other than the three traditional monolignols have now been implicated in the lignins in a variety of natural, mutant, and transgenic plants [1, 2, 4-7, 22-24]. Thus, dihydro-hydroxycinnamyl alcohols and their peroxidase-H₂O₂-derived arylpropane-1,3-diols are found in softwood lignins and beyond. Arylglycerols, once thought to be artifacts of ball-milling (from cleavage of β -ethers) are now suspected as authentic monomers. A range of acylated monolignols, notably monolignol γ -acetates, γ -hydroxybenzoates, and γ -*p*-coumarates are now known to be lignin precursor 'monomers' (monomer conjugates). Secoisolariciresinol (a lignan-like product resulting from reductive steps post- β - β -dehydrodimerization) also appears to be implicated in softwood lignins. Ferulates on arabinoxylans actively incorporate into lignins in grasses and could therefore be considered monomers [25-28]. Recently, the flavone tricetin has also been implicated in grass lignins [29]. Ferulates and tricetin appear to act as nucleation sites for beginning the growth of a lignin polymer chain.

There are the whole array of monomers that plants appear to export to the wall when their ability to biosynthesize their monolignols becomes compromised. In each case, these can then be regarded as monolignol replacements; Lewis' contention years ago that monomer replacement would not be tolerated [30] has been repeatedly diminished as ever more are discovered [1]. One classic is 5-hydroxyconiferyl alcohol in COMT-deficient plants, where its incorporation into lignin results in novel cyclic benzodioxane units in the polymer [31-38], often in very high amounts – we find that 90% of the measurable interunit linkage types in a high-5-hydroxyconiferyl alcohol lignin, produced in F5H-up-/COMT-down-regulated transgenic Arabidopsis, are benzodioxanes [38, 39]. Another classic is the hydroxycinnamaldehydes that are shown to fully incorporate into lignins in CAD-deficient dicots [34, 40-42], and in a more minor way in CAD-deficient softwoods [43, 44]; low levels are likely incorporated into all natural lignins. The derived hydroxybenzaldehydes are also implicated in normal and transgenic plants. Mutants and transgenics with extremely high hydroxycinnamaldehyde levels are beginning to show up in research labs. More recently, evidence has been provided that ferulic acid itself incorporates to a small degree into lignins in CCR-deficient plants, producing novel acetal linkages in the polymer backbone (as revealed by marker compounds from thioacidolysis, and evidenced in NMR spectra) [45, 46].

Until recently, the 'missing hydroxycinnamyl alcohol,' caffeyl alcohol (also potentially available from the monolignol biosynthetic pathway) had not been authenticated in lignins. It was first detected in a CCoAOMT-deficient pine system [47], but never in dicots, even those deficient in either/both OMTs, CCoAOMT and COMT. Recently, however, a seed coat polymer, that we decided to go ahead and call a lignin, was found to be 100% derived from caffeyl alcohol [48]; the seed coat was from vanilla beans, and was 80% lignin (again, entirely this new C-lignin). Since then, other plant seed coats, in both monocots and dicots, have been found with C-lignins [49]. More recently, a few plants have been found with mixed S/G and C lignins, but the two polymers appear to derive from independent polymerization – the C-polymer has no detectable connection to the G/S polymer. A plant tissue in which the lignin is derived entirely from 5-hydroxyconiferyl alcohol may also have been discovered. There are other monomers implicated in lignification.

'New' Monomers

As a result of the above observations (of lignin monomer replacement) it has become somewhat fashionable to consider incorporating other novel monomers into lignins. A review of some interesting possibilities has been published [2]. Several new approaches to altering lignins have already been targeted with some success.

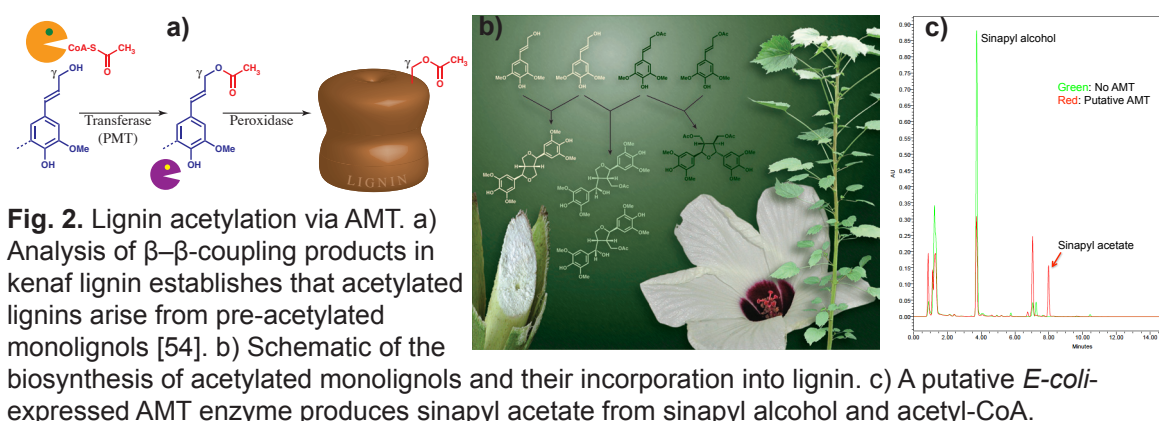
In some of the cases above, and in a rather promising new approach to altering lignins, the genes implicated or required were not known. Here we describe ‘new’ transferases that are required for the introduction of various monolignol ester conjugates into lignins.

‘NEW’ TRANSFERASE GENE 1: *AMT*

A number of plants have a high degree of natural lignin acetylation. Kenaf has ~60% of its lignin units acetylated [50], but more recently some plants have been shown to have even higher levels [51-53]. In all cases, the acetate is entirely (or at least very predominantly – acetates can migrate) on the γ -position of the lignin sidechain. It has now been rather compellingly shown that this acetylation results from the plants’ use of monolignol acetate conjugates in lignification [54, 55]. Usually, but not universally, the major acetylation is on syringyl units, implicating sinapyl acetate over coniferyl acetate as the primary monomer conjugate. Such acetylation is often missed, in part due to the past predilection for running NMR spectra of lignins as their peracetates, or because the used analytical methods either cleave such esters or, such as in the case of the standard DFRC method, acetylate all products during the procedure, masking the presence of any natural acetates. A modification of the DFRC method allows for natural lignin acetylation to be revealed, and for the levels of acetylation (at least on the releasable monomers) to be quantified [54, 56]. Most recently, re-evaluation of grasses is showing that their lignins, known previously to be acylated by *p*-coumarate (see below), are also partially acetylated [29, 57].

We have termed the transferase gene involved as coding for an **acetyl-CoA:monolignol transferase (AMT)** enzyme. The gene(s) is (are) currently unknown, although a candidate or two are now in hand. Without identifying that gene here, the activity of the expressed AMT enzyme is shown in Figure 2. It takes the monolignol, in this case coniferyl alcohol, and acetyl-CoA and produces the γ -acetylated monolignol.

The role of such lignin acetylation is currently unknown. It is hoped that having the gene, and up- and down-regulating it in various plants that either do not or do have lignin acetylation, might provide some insight. It is considered a useful gene in the quest to improve biomass for saccharification and fermentation; pretreatments often release acetate from plant cell walls, including the lignin component, that can interfere particularly with the fermentation of cell-wall-derived sugars to ethanol and other liquid biofuels.



'NEW' TRANSFERASE GENE 2: *PMT*

As noted in the introduction, it is well known that all grasses, both C3 and C4 grasses, have lignins that are acylated by *p*-coumarate [4, 6, 7, 25, 26, 29, 52, 57-60]. In fact, *p*-coumaroylation of lignins appears to now also extend beyond the grasses! Since the initial NMR experiments that revealed that *p*-coumarates acylated exclusively the γ -OH of lignin sidechains in corn [59], it has been established that such acylation is via lignification with monolignol *p*-coumarate ester conjugates. The DFRC method, which rather uniquely cleaves β -ether bonds in lignin but leaves esters intact, has been particularly valuable for determining that, firstly, *p*-coumarates do acylate the γ -OH of lignin sidechains, and secondly that they are primarily on syringyl units in most grasses, wheat being one exception [29, 61]. Analytical thioacidolysis also reveals lignin *p*-coumaroylation; it is not however known the extent to which the ester survives vs is cleaved during the procedure [62].

The *PMT* gene encoding the presumed *p*-coumaroyl-CoA:monolignol transferase (*PMT*) was unknown. *PMT* enzyme activity was demonstrated in corn protein extracts [63], and recently, a likely candidate *PMT* gene was gained from mining the rice genome [64]. The *E. coli*-expressed *PMT* protein showed the appropriate activity, taking any of the three monolignols and *p*-coumaroyl-CoA and producing the monolignol *p*-coumarate conjugates, Figure 3. That this gene/protein has this role *in planta* is just now being established in the model grass *Brachypodium*, where the gene-homolog has now been successfully downregulated and a mutant may have been identified.

Again, the role of *p*-coumaroylation is unknown. It was suggested, and has been demonstrated, that *p*-coumarates are excellent as radical-transfer agents for such a possible role in lignification [65-67]. However, although the ability to 'catalyze' the dimerization (and, by extension, polymerization) of sinapyl alcohol, via radical transfer from the readily 'radicalized' *p*-coumarate to the only slowly 'radicalized' sinapyl alcohol, as been shown, it is far from clear that this is its role *in planta*. The benefit of having *p*-coumarate end-units (they are essentially all free-phenolic pendant units, and are not coupled into the polymer backbone) on the lignin polymer is also unclear. Again, it is hoped that having the gene might provide some insight into its function, as well as delivering the potential to lower the amount on biomass crop grasses – *p*-coumarate released from grass biomass during pretreatment can inhibit microbial fermentation of cell-wall-derived sugars. Another major reason that our groups are interested in the *PMT* gene is that it is an analog of other genes of interest, BMT and FMT – see below.

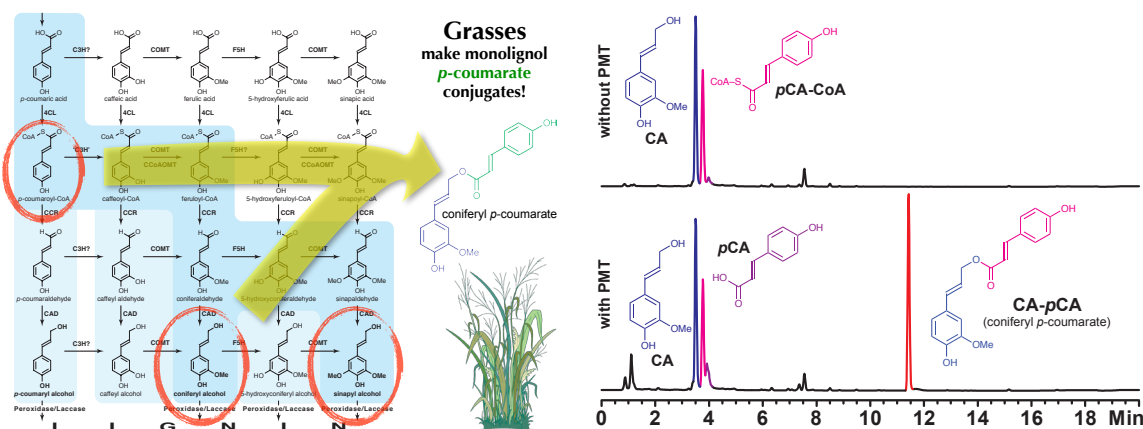


Fig. 3. Lignin *p*-coumaroylation in grasses via *PMT*. Left: Monolignols are, in principle at least, acylated via pathway intermediate *p*-coumaroyl-CoA. Right: An *E. coli*-expressed *PMT* enzyme produces conferyl *p*-coumarate from conferyl alcohol and *p*-coumaroyl-CoA.

‘NEW’ TRANSFERASE GENE 3: *BMT*

Several plants also have lignins acylated by *p*-hydroxybenzoate. Thus willow (*Salix*), poplar/ aspen (*Populus*), and various palms (*Palmae*) all have *p*-hydroxybenzoylated lignins [4, 68], the level being particularly high in oil palm empty fruit bunches [69, 70]. The presumed *p*-hydroxybenzoyl-CoA:monolignol transferase (*BMT*) and its associated *BMT* gene remain currently unknown. Again, the role of such *p*-hydroxybenzoylation, which is almost exclusively on syringyl units (Ralph, unpublished), is also unknown. The gene is sought for similar reasons as its analog, the *PMT* gene.

‘NEW’ TRANSFERASE GENE 4: *FMT*

A major aim of much current research is to alter lignin composition/structure such that pretreatment is more efficient under milder conditions. [Reducing lignin levels in plants remains an aim of some research groups but is not without obvious limitations]. An exciting approach stems from the idea of engineering more readily cleavable bonds into the backbone of the lignin polymer [2, 25, 71]. The idea, of course, is that the currently most readily cleavable bond in lignin, the β -ether, still requires $\sim 170^\circ\text{C}$ in NaOH or $\sim 200^\circ\text{C}$ in acid for an hour or more to significantly cleave the polymer and effect delignification. If simple ester bonds, for example, could be engineered into the lignin polymer backbone, molecular weight reduction and consequent delignification could be accomplished considerably more easily, reducing the energy demand and cost of pretreatment (which remains the highest cost step in cellulosic ethanol production from biomass, for example).

We have reviewed how several classes of plant-derived phenolics could be conceivably incorporated into lignins to facilitate delignification [2]. Introducing readily cleavable bonds into the polymer is a promising approach. Several methods exist, some, including the introduction of rosmarinic acid and catechin gallates as monomers, have been validated on a model basis [72-74]. One of the most promising ideas, that of lignifying with monolignol ferulates, is seen as being a particularly attractive approach, with proof of principle having been established via a cell wall model system [25, 71]. The idea is simple, Figure 4. If monolignol ferulates can be biosynthesized and sent to the cell wall, they are known to be compatible with the coupling and cross-coupling reactions of lignification; ferulate esters to grass arabinoxylans incorporate integrally into

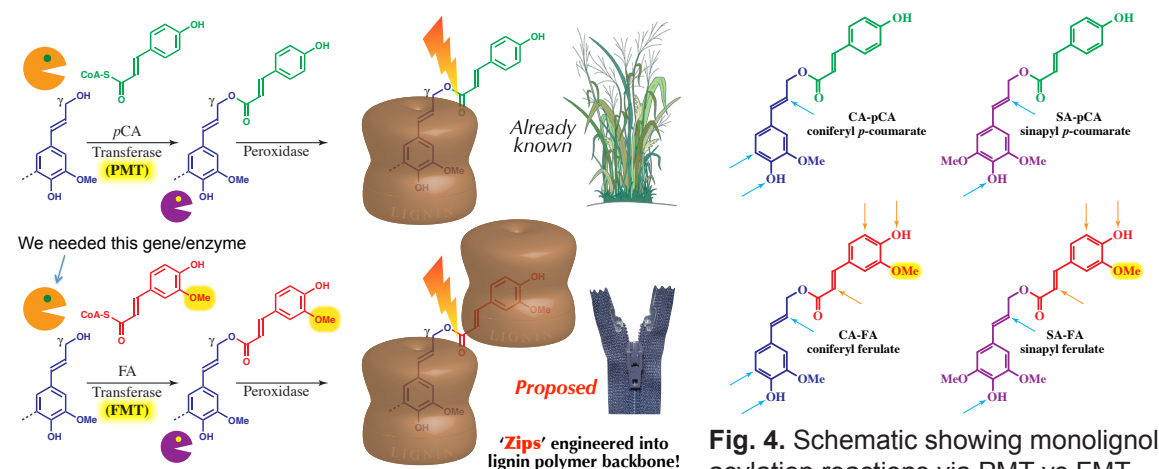


Fig. 4. Schematic showing monolignol acylation reactions via PMT vs FMT.

grass lignins – their dehydrodimers also incorporate. Incorporation of monolignol ferulates into lignification thereby produces lignins that have ester linkages in the polymer backbone, Figure 4.

However, this lignin modification is very different from all those tried to date. It requires an activity that is not present in the known pathway. That is, it requires a transferase that attaches ferulate to monolignols. Such a feruloyl-CoA:monolignol transferase (FMT) is not present in the monolignol biosynthetic pathway, in any known plant, and therefore must either be developed, e.g., by directed evolution from other pathways/enzymes/genes, or must be sought *de novo* from other plant pathways and be introduced into the lignin monomer pathway. The entire idea of utilizing a complex monolignol conjugate, something that looks rather unlike a simple monolignol, would be a patently absurd idea were it not for the queues from nature – that entirely analogous conjugates, monolignol *p*-coumarates (lacking only the monolignol ferulate's methoxyl on the hydroxycinnamate) are already utilized for lignification in all grasses (and perhaps beyond).

The idea therefore is to 'find' the required enzymatic activity, secure the gene, attempt to introduce it into the monolignol pathway by (hopefully as simply as) tethering it to a xylem-specific promoter, and hope that the conjugate will be transported to the cell wall. If it is, given the solely chemical nature of the lignification reaction, polymerization involving un-aided radical coupling reactions, there is sufficient evidence to fully expect that the monolignol conjugates would be used for lignification and would therefore become integrally incorporated into the lignin polymer (thereby introducing the lignin 'zip' – the readily cleavable bond – into the lignin backbone).

Finding an FMT gene

Evidence for the required FMT enzyme activity is in at least three plants. Both kenaf and balsa have ferulate-containing extractives with structures that logically derive from dehydrodimerization of monolignol ferulates and close derivatives. Both can be shown to have the required FMT activity in their protein extracts. Another plant, the Chinese medicinal *dong quai*, or Chinese angelica (*Angelica sinensis*) produces the conjugate coniferyl ferulate itself in its roots, at a level of up to ~2% of the dry root mass. It was therefore the latter that was used first to identify the gene. Deep sequencing of *Angelica sinensis* has now revealed a candidate FMT gene; the *E-coli*-expressed FMT enzyme has exactly the properties required. That is, it makes monolignol ferulate conjugates when fed monolignols and activated ferulate (feruloyl-CoA) as substrates, Figure 5. Importantly, it does not efficiently produce monolignol *p*-coumarate conjugates when *p*-coumaroyl-CoA is a substrate; as noted above, this does produce conjugates that are used in lignification (in grasses) but does not result in ester bonds in the lignin backbone because the *p*-coumarate moiety does not become involved in the radical coupling reactions of lignification.

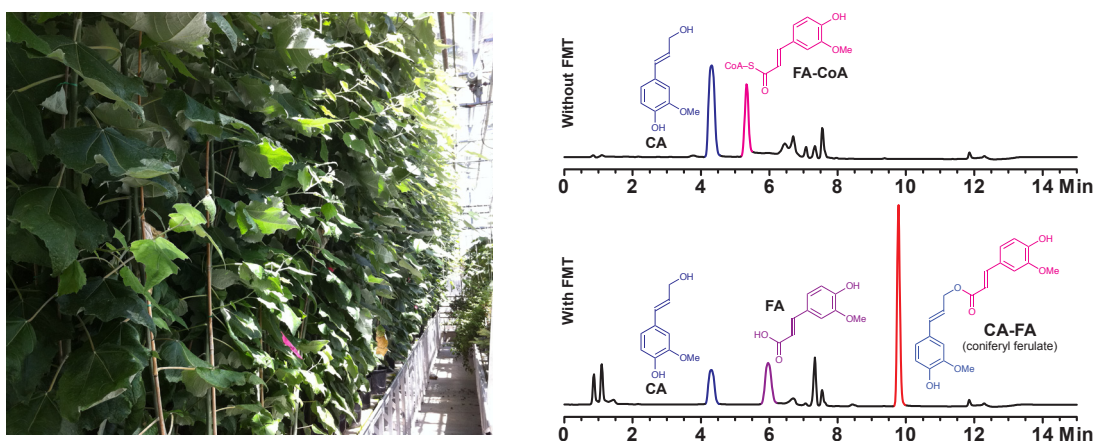


Fig. 5. Introducing monolignol ferulates into lignification via FMT. Left: Picture of transgenic poplars at UBC. Right: An *E-coli*-expressed FMT enzyme successfully produces coniferyl ferulate from coniferyl alcohol and feruloyl-CoA.

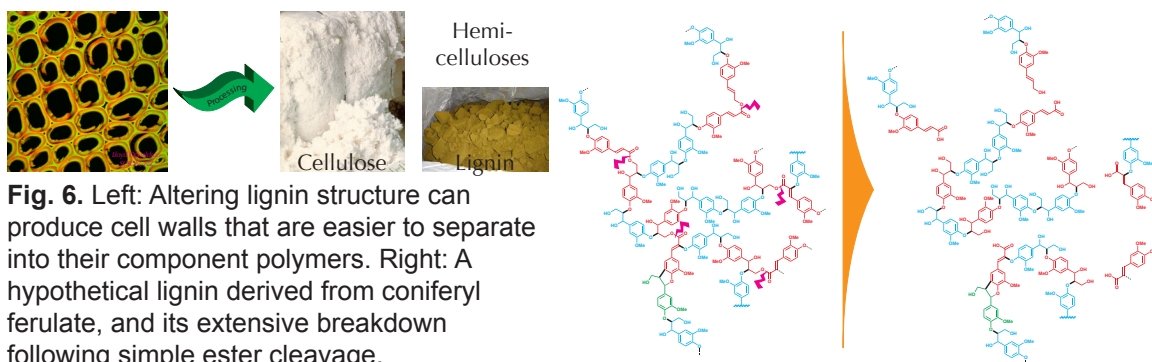


Fig. 6. Left: Altering lignin structure can produce cell walls that are easier to separate into their component polymers. Right: A hypothetical lignin derived from coniferyl ferulate, and its extensive breakdown following simple ester cleavage.

Producing FMT Transgenic Plants and Altering the Lignin

The next step is to introduce the gene into (model) plants that do not already possess this activity. Using a universal promoter, the FMT gene has been introduced into poplar, arabidopsis and Brachypodium where, in all cases, it has been shown to express active FMT protein. More recently, we appear to have been successful in introducing the FMT gene into poplar using a xylem-specific promoter; preliminary experiments suggest that such plants are biosynthesizing the monolignol ferulate conjugates and, more importantly, sending them to the wall for lignification. We hope to be able to present this evidence by the time of the 2013 ISWFPC meeting!

Ramifications

If success is validated, lignin modification in plants will be on the precipice of a bold new direction [25]. It will mean that it is indeed possible to introduce entirely new phenolic monomers (monolignol conjugates, in this case), from beyond the normal pathway, into the lignification process, and to radically engineer the nature and chemical properties of that lignin polymer. It is not too much of an exaggeration to note that these plants will have then been actually 'designed' for processing. i.e., that the plants retain lignins that continue to function appropriately in the plant, but that have been structurally augmented in such a way that plants containing them are much easier to pretreat and/or delignify. This is something of a holy grail for lignin modification that could/should have profound implications for pulping and biomass conversion to biofuels. In particular, it should allow for pulping or pretreatments to be carried out under significantly milder conditions, improving the energy balance of the processes, and reducing costs. Indeed, it is conceivable that the zip-lignin trait is so compelling that any plant destined for pulping, or pretreatment to produce sugars for the 'sugar platform' biofuels, should contain this gene!

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